Nucleolin Regulation and Targeting in Skin Cancer

Undergraduate Research Thesis

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By

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Abbreviations

3, 3', 5, 5'-Tetramethybenzindine	TMB
Ak strain transforming	AKT
Arg-Gly-Gly	RGG
Ataxia telangiectasia mutated	ATM
Basal Cell Carcinoma	BCC
B-cell lymphoma/leukemia 2	BCL-2
Bovine serum albumin	BSA
Casein Kinase 2	CK2
DiGeorge Syndrome Critical Region 8	DGCR8
Dulbecco's Modified Eagle Medium	DMEM
Dulbecco's Phosphate Buffered Saline	PBS
Enzyme-linked immunosorbent assay	ELISA
Fetal Bovine Serum	FBS
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Glycine arginine rich	GAR
Guanosine triphosphate	GTP
Horseradish peroxidase	HRP
Kilodalton	kDa
MicroRNA	miRNA
Mouse double minute 2	MDM2
Mre11-Rad50-Nbs1	MRN
Non-Melanoma Skin Cancer	NMSC

Non-Transfected	NT
Nucleolin	NCL
Nucleotide	nt
Phosphatase and Tensin Homologue	PTEN
Precursor microRNA	pre-miRNA
Primary microRNA	pri-miRNA
Ribosomal RNA	rRNA
RNA-Binding Domain	RBD
RNA Induced Silencing Complex	RISC
RNA Recognition Motif	RRM
Single Chain Variable Fragment	scFv
Squamous Cell Carcinoma	SCC
Standard Deviation	SD
Tris phosphate buffered saline	TPBS
Ultra-Violet Radiation	UVR
Ultra-Violet A	UVA
Ultra-Violet B	UVB
Ultra-Violet C	UVC
Variable Heavy	V_H
Variable Light	V_L
Wild Type	WT

Abstract

Skin cancer is the most frequently diagnosed malignancy in the United States, and the more aggressive forms suffer from limited or ineffective treatment options. These cancers are commonly caused by damage to the DNA from exposure to UV-radiation via sunlight. In these cancers, nucleolin (NCL) is frequently found overexpressed and localized to the cell surface, however, little is known about the mechanism of NCL upregulation in cancer cells. NCL is involved in the regulation of the biogenesis of a specific set of oncogenic microRNAs, including miR-21, -221, and -222, which contribute to cell survival and proliferation and are also found overexpressed in skin cancers. Targeting surface NCL has shown promise as a potential therapy for several cancers, and for this reason, we developed a single chain variable fragment, called 4LB5, which binds NCL with high affinity and inhibits its microRNA processing abilities in breast cancer cell lines.

This study aimed to determine a mechanism of NCL overexpression in skin cancer and validate the use of 4LB5 as a tool for skin cancer therapy. First, we investigated the regulation of NCL by microRNAs, because these molecules have been widely implicated in the regulation of proteins important to the development of cancers. Direct targeting of NCL by miR-203 was demonstrated through luciferase assays and miR-203 transfections. To further understand this regulatory mechanism and to link NCL expression to the primary cause of skin cancer, DNA damage, we investigated the regulation of miR-203 by p53. P53 is a crucial player in the cellular response to stress and functions to maintain genomic integrity by regulating DNA repair, cell cycle progression, and apoptosis. Additionally, it is one of the most frequently mutated genes in cancer. When exposed to UV-radiation, skin cancer cell lines increased p53 expression, and we observed a corresponding rise in miR-203 levels. Conversely, knockdown of p53 through transfection with

si-p53 led to a reduction in miR-203. From this, we propose that NCL expression is, in part, regulated by p53 via miR-203. When p53 is inactivated or mutated, expression of miR-203 is impaired leading to excessive NCL production.

By treating skin cancer cells with 4LB5, we hypothesized that we could reduce cellular proliferation by counteracting the upregulation of NCL. Through ELISA, we showed that 4LB5 specifically binds to cell surface NCL in skin cancer cell lines. To confirm that 4LB5 had antineoplastic effects in skin cancer, we first assessed the effect of 4LB5 treatment on the NCL-associated microRNAs, miR-21, -221, and -222. Through Real-Time PCR we demonstrated that 4LB5 treatment resulted in reduced levels of all three microRNAs. Western blot confirmed that down-regulation of these microRNAs resulted in an upregulation of the tumor suppressor PTEN and a decrease in activated AKT. Finally, by alamarBlue and colony assay, we observed a reduction in cellular proliferation in treated melanoma cell lines.

In summary, here we uncovered an unknown mechanism in skin cancer that is responsible, at least in part, for the upregulation of NCL seen in these malignancies and provided support for 4LB5 as a tool for the treatment of skin cancer.

Introduction

Skin Cancer. Skin cancers account for most cancer diagnoses in the United States and represent a significant and increasing burden on the health care system. It is estimated that in the United States one in five people will develop skin cancer. From 2002 to 2011, the average annual total cost associated with skin cancer rose $4.5 billion dollars, an increase in cost of 126.2% (1). During the same period, the cost for all other cancers increased by only 25.1% (1). Rates of skin cancer diagnosis increase significantly with age, reflecting a long separation between exposure to a carcinogen and the onset of cancer.

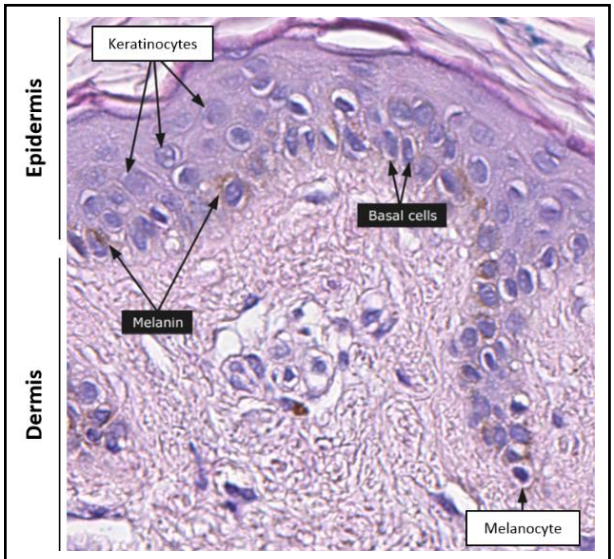

Figure 1. Keratinocytes and melanocytes in the skin. Keratinocytes are the predominant cells in the epidermis. The basal layer of the epidermis is comprised of basal cells which constantly divide to replace dead and damaged keratinocytes in the upper layers (6). Melanocytes, the melanin pigment producing cells, also reside in the basal layer. These cells deliver melanin to keratinocytes through dendrites. Figure adapted from The Human Protein Atlas (84, 85).

Skin cancers are divided into categories based on clinical behavior and the cells from which the malignancy originates. The two major groups are non-melanoma skin cancers (NMSC) which arise from keratinocytes - the predominant cells in the epidermis - and cutaneous malignant melanomas which form from the melanocytes - the pigment producing cells of skin and hair (Fig. 1) (2).

NMSCs account for the vast majority of skin cancer diagnoses, with about 1.3 million cases reported in the U.S. each year (3), and the rate of diagnosis is expected to double in the next 30 years (4). While common, NMSCs have a good long-term prognosis due in part to the tendency of these malignancies to remain within the primary disease site. The two main forms of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Accounting for roughly 80% of all NMSCs and 30% of all cancer diagnoses (3), BCCs typically arises from the basal layer of

the epidermis (2). These tumors are most frequently found on areas of the body that undergo constant sun exposure, such as the head and neck, but can also form on the trunk and extremities (2). BCC is rarely fatal and because of its low risk of metastasis, treatment through surgery is generally sufficient to remove the malignancy (2). SCCs account for roughly 16% of all skin cancers and are considered the second most common form of skin cancer (2). These tumors are more aggressive than BCCs, more likely to metastasize, and account for most NMSC-related metastatic disease and death (5). Additionally, SCC is considered one of the top five most costly cancers in the US with incidence expected to rise in the future due to increasing sun exposure, age, and public awareness of skin cancer (5).

Cutaneous malignant melanoma, accounts for only 4% of skin cancer diagnoses, but is responsible for roughly 75% of skin cancer related fatalities (2). In 2010, about 68,000 new cases of melanoma were diagnosed, and in the same year, 8,700 deaths were attributable to melanoma in the United States. When detected early, surgical excision alone can be sufficient to remove the malignancy, however, in the case of advanced disease, long-term prognosis is poor due to the fast invading and metastasis prone nature of these tumors (6).

Risk factors for melanoma include fair pigmentation, poor tanning ability, multiple nevi, clinically atypical nevi, dysplastic nevi, and freckling (6, 7, 8). One of the greatest risk factors for the development of skin cancer is exposure to ultra-violet radiation (UVR) via sunlight (6). Through direct and indirect mechanisms, such as gene mutation, formation of pyrimidine dimers, immunosuppression, and oxidative stress, UVR is able to damage skin cells (3). UVR is divided into three types based on wavelength and energy: ultraviolet A [UVA (315-400 nm)], ultraviolet

B [UVB (280-135 nm)], and ultraviolet C [UVC (100-280 nm)]. As shown in Fig 2, UVR distribution in the skin varies with type and depth. UVA radiation accounts for 90-99% of all UVR that reaches the Earth's surface and contributes to skin aging and tanning. Excessive and long-term exposure to UVA is believed to be harmful (3) and an important factors in the development of melanoma (9, 10). UVB accounts for 1-10% of UVR and is responsible for causing sunburns, wrinkling, tanning, and NMSCs. Often, UVB can create mutations in the p53 gene, a transcription factor involved in DNA repair and cellular apoptosis, which leads to dysregulation of these processes and the initiation of skin cancer (11).

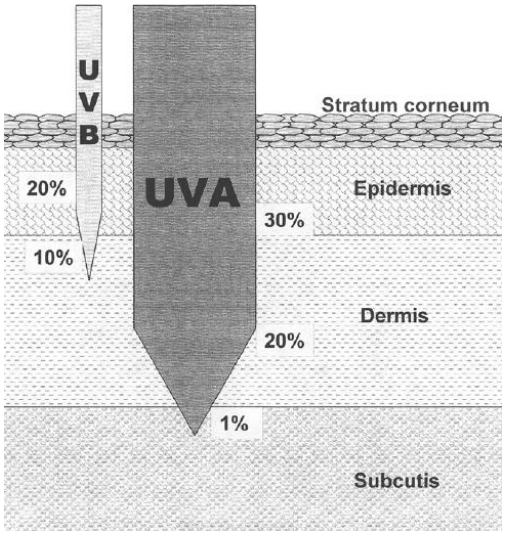

Figure 2. Penetration of UV radiation in the skin. Of the UVR to reach the Earth's surface, 90% is UVA while only about 10% is UVB. UVA light reaches the deeper layers of the skin and is believed to be important in the development of melanoma. UVB light can cause DNA damage in the form of pyrimidine dimers. (17)

P53. P53 was first classified as an oncogene, but later work determined that wild-type p53, encoded by the *TP53* gene, suppressed growth and oncogenic transformation of cells in culture (12). Since its initial discovery, p53 has become known as the 'guardian of the genome' because of its critical roles in DNA repair and damage related cell death. As a DNA binding protein, p53 functions as a transcription factor to regulate the expression of target genes at the transcriptional level (13). In response to cellular stress, p53 is activated to regulate a wide range of pathways and processes to maintain genomic integrity and prevent tumorigenesis. P53 is comprised of two N-terminal transactivation domains followed by a conserved proline-rich domain, a central DNA binding domain, and a C-terminus containing a nuclear localization signal. For transcriptional

activity, p53 contains an oligomerization domain (12). The majority of mutations to p53 found in tumors are to the DNA binding domain encoding region of *TP53* (13, 14).

In the absence of cellular stress, p53 protein levels are kept low by interaction with the E3-ubiquitin protein ligase MDM2. This binding leads to the proteasome-mediated degradation of p53 in the cytoplasm (13, 15). In response to cellular stress, p53 and MDM2 undergo post-translational modifications dependent on the stress type by a variety of enzymes. Specifically in response to DNA damage, p53 is phosphorylated by ATM resulting in the stabilization of p53 and its release from MDM2 (16). Once activated, p53 forms a quadraplex that regulates the expression of target genes by binding in a sequence specific manner to DNA which allows it to modulate cellular processes including cell cycle arrest, apoptosis, senescence, and DNA repair. Some of these process are summarized in Fig. 3.

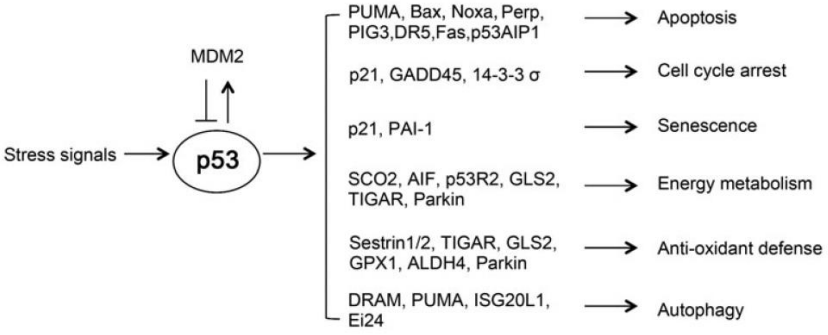

Figure 3. P53 functions as a tumor-suppressor in multiple cellular pathways. In the absence of cellular stress, interactions with MDM2 keep p53 levels low. When under stress, p53 is activated and released from MDM2 via post-translational modifications. In its active form, p53 regulates the expression of numerous genes involved in cellular processes including apoptosis, cell cycle arrest, and senescence. (13)

TP53 is one of the most commonly mutated genes in human cancers. In skin cancer specifically, inactivating mutations to p53 are common, and UV-induced mutations to *TP53* have been implicated as an important step in the development of these cancers. *TP53* mutations are present in the majority of NMSCs (90% in SCC and 50% in BCC) (17). The majority of p53

mutations in cancer are missense mutations which result in the expression of a full length mutant protein (13). When wild-type and mutant p53 are present in the same cell, mutant p53 can inactivate the wild-type protein through a dominant negative effect (18), but often, p53 mutations are followed by a loss of heterozygosity in the tumor leading to a complete absence of wild-type p53 (13). In some cases, p53 mutants gain new oncogenic functions in the cell. For instance, mutant p53 can regulate the expression of specific microRNAs, short RNA molecules involved in the regulation of gene expression, leading to new oncogenic functions. In endometrial cancer, mutant p53 can directly interact with the miR-130b promotor and inhibit its transcription leading to the promotion of the epithelial-mesenchymal transition and cancer cell invasion (19). In breast cancer, mutant p53 induces miR-155 expression which drives the invasion of cancer cells (20). Mutant p53 has also been shown to interact with Drosha to inhibit the processing of certain primary microRNAs (21).

Nucleolin. Nucleolin (NCL) is one of the most abundant nucleolar proteins, accounting for as much as 10% of total protein in the nucleolus (22). While its predicted molecular mass is 77 kDa, NCL is described as a 100-110 kDa protein due to the amino acid composition of the N-terminus and the addition of post-translational modifications (22). NCL is composed of three major domains: the N-terminal domain, the RNA binding domains, and the GAR region (Fig. 4A). The N-terminal domain is composed of highly acidic regions separated by basic sequences that allow the protein to interact with histone H1 and modulate DNA condensation in chromatin (23, 24, 25). This region is also involved in protein-protein interactions with ribosomal proteins. The N-terminus is highly phosphorylated by kinases like the casein kinase 2 (CK2) which probably regulates NCL function during the cell cycle (22). The central region of NCL is composed of

RNA-binding domains (RBDs). In animal cells, NCL contains 4 RBDs while yeast and plant NCL contains 2 RBDs (23). Through the RNA recognition motifs (RRMs) in the RBDs, NCL interacts with the stem loop structure of RNA to participate in the modification and processing of pre-ribosomal RNA (23). The third domain, the GAR (or RGG) domain is a region rich in Arginine-Glycine repeats in the order Arg-Gly-Gly (22). This region functions in protein-protein interactions and has been implicated in ribosomal assembly and nuclear import of ribosomal proteins (23, 26).

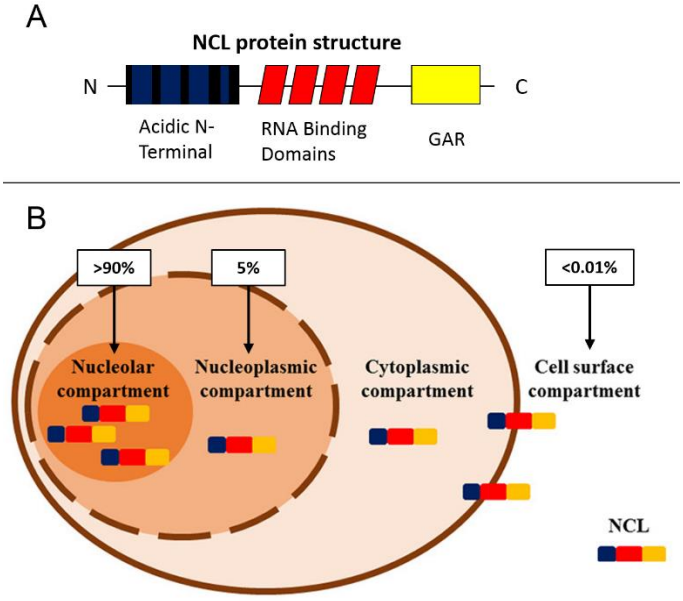

Figure 4. Structure and localization of NCL. (A) Nucleolin is composed of three major domains: the N-terminus, centrally located RNA binding domains, and the glycine and arginine rich GAR domain. (B) The majority of NCL is located within the nucleolus, but NCL is also found throughout all the major cellular compartments. Percentages indicate the fraction of total NCL found in each compartment. In cancer cells, NCL is found on the cell surface, however, where and how it is incorporated is unknown. Adapted from (87)

The majority of NCL is located within the nucleolus, but it is also present in other nuclear regions, the cytoplasm, and on the plasma membrane (Fig. 4B) (27), however, where and how NCL is incorporated on the membrane is unclear. The principal function of NCL is ribosomal RNA (rRNA) processing and ribosome biogenesis where it plays key roles in rRNA transcription, rRNA maturation, and ribosome assembly (22, 23, 27). In addition to its function in ribosome biogenesis, NCL has been implicated in numerous other biological processes throughout the cell. Roles for NCL have been established in DNA metabolism where it affects DNA replication, repair, recombination, telomere maintenance, and chromatin remodeling. During the DNA damage response, NCL has been shown to co-localize with sites of DNA double strand breaks and interact

with Rad50 and the MRN complex to facilitate H2A-H2B removal at transcription loci (28, 29, 30). In response to UV-induced DNA damage, NCL has been shown to interact with proteins involved in nucleotide excision repair (30). NCL is involved in nucleocytoplasmic transport, likely moving newly assembled ribosomal subunits into the cytoplasm. The stability and translation of specific subsets of mRNAs can be affected by NCL through interactions with AU-rich elements in the 5'-UTR or through interactions with G-rich sequences throughout the 5'-UTR, coding region, or 3'-UTR. NCL can either increase or decrease the half-life of mRNA through interactions with the UTRs of target molecules. *Bcl-2* mRNA which encodes the anti-apoptotic and pro-oncogenic protein BCL-2, is stabilized by NCL through an interaction between the protein and the AU-rich elements in the mRNA (31, 32). By binding with the 5'-UTR of *TP53* mRNA, NCL can inhibit the induction of p53 in response to DNA damage (33). NCL also regulates the biogenesis of a specific subset of microRNA molecules through interactions with the microprocessor complex (34).

MicroRNAs. MicroRNAs (miRNAs) are small, noncoding RNA molecules of about 22 nt that function as regulatory elements by base pairing with sequences in mRNA molecules (35). The first miRNA, *lin-4*, was discovered in *Caenorhabditis elegans* over 30 years ago (36). Since their initial discovery, miRNAs have been implicated in numerous human diseases. Gene expression and profiling studies have revealed that miRNAs are frequently dysregulated in human diseases, and in several cancer types alterations in miRNA expression patterns have been identified.

All miRNAs undergo a biogenesis process that converts the primary miRNA transcript into the mature, functional miRNA (Fig. 5). This process begins with the transcription of a miRNA gene. These genes are located throughout the genome and may be incorporated into an intron or

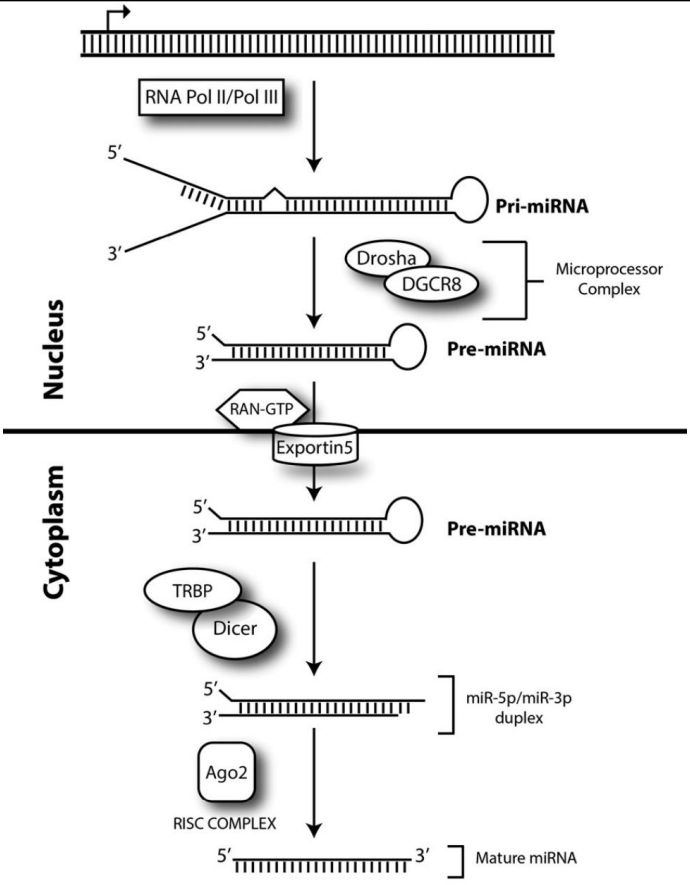

Figure 5. MicroRNA processing. In the nucleus, a miRNA gene is transcribed by RNA polymerase II/III resulting in a primary-miRNA (pri-miRNA) transcript containing a precursor stem loop structure. The stem-loop precursor (pre-miRNA) is released after cleavage by the microprocessor complex. In the biogenesis of specific miRNAs, NCL interacts with this complex to regulate miRNA processing. The pre-miRNA is exported into the cytoplasm where further processing by Dicer and RISC convert the double stranded pre-miRNA into a 22 nt mature miRNA. (35)

untranslated region of a protein coding gene or present as an independent gene (35). Transcription of a miRNA gene by RNA polymerase II results in a primary miRNA (pri-miRNA) transcript with a stem-loop precursor RNA structure (37). Generally, the pri-miRNA is spliced, capped, and polyadenylated in a manner similar to protein coding mRNAs. The first endonuclease processing step occurs during or subsequent to transcription and is carried out by the enzyme Drosha and its associated RNA binding protein DGCR8 which is required for cleavage of the pri-miRNA (38, 39, 40). Cleavage by Drosha releases the stem-loop precursor from the pri-miRNA transcript sequences to form the precursor miRNA (pre-miRNA) (41). The pre-miRNA is exported out of the nucleus by Exportin5 in a Ran-GTP dependent manner (42). In the cytoplasm, the second processing step is completed by Dicer, an endonuclease, which cleaves the loop region of the pre-miRNA to release the mature miRNA in a duplex form of about 21 nt (43, 44). One strand of the duplex is loaded into the RNA induced silencing complex (RISC) while the other strand, denoted as the star strand, is typically degraded (45, 46). However, some star strands are loaded into the RISC at a similar frequency as the mature miRNA. In these cases, the strand from the 5’ end is labeled 5p and the strand from the 3’ end is

labeled 3p (47). In the RISC, the protein Argonaute directly binds the mature miRNA which serves as a guide to target mRNAs leading to the destabilization and degradation of these mRNAs (47).

During miRNA processing, NCL has been reported to interact with the microprocessor complex to regulate the biogenesis of a specific subset of miRNAs including miR-21, miR-221, and miR-222 (34, 48). Aberrant expression of miR-21 was first observed in human glioblastoma, and it has since been found overexpressed in numerous cancers including breast, colon, lung, pancreatic, prostate, stomach, and head and neck cancers (49, 50, 51). Overexpression of miR-21 in hepatocellular cancer cells led to an increase in cellular proliferation and migration (52). Conversely, inhibition of miR-21 reduced invasion, anchorage-independent colony formation, and proliferation in these cells (52, 53). Additionally, overexpression of miR-21 has been observed in melanoma tissues where it is associated with increases in proliferation and decreases in apoptosis (54). Among other proteins, miR-21 regulates the expression of the tumor suppressor protein phosphatase and tensin homologue (PTEN) and downstream PI3-kinase signaling (52) leading to the activation of AKT (55). Similar to miR-21, miR-221 and miR-222 are found overexpressed in certain cancers, including metastatic melanoma, where increased proliferation and cellular migration were observed in cells with enhanced expression of these miRNAs (56).

MicroRNA-203 is the most abundant keratinocyte-specific miRNA in the epidermis (57) and is required for skin development and differentiation. It plays an anti-proliferative role in the skin by targeting p63, a transcription factor important for maintaining basal keratinocyte proliferative potential in the epidermis (58). Downregulation of miR-203 promotes the epithelial-mesenchymal transition and enables tumors to acquire metastatic features (59, 60). In keratinocytes, miR-203 expression is dependent on p53 levels (61).

Nucleolin Targeting, scFv, and 4LB5. NCL is an attractive therapeutic target because of its oncogenic role and its cancer cell specific localization to the plasma membrane. Several approaches to target cell surface NCL have been explored. AS1411, a DNA aptamer against NCL, was the first nucleolin targeting agent to reach human clinical trials and has shown promise as a potential antineoplastic treatment (62). In phase I and II trials AS1411 showed low toxicity and evidence of therapeutic activity, but suffered from a low overall response rate among patients (63, 64). Other groups have explored targeting NCL through peptides (65).

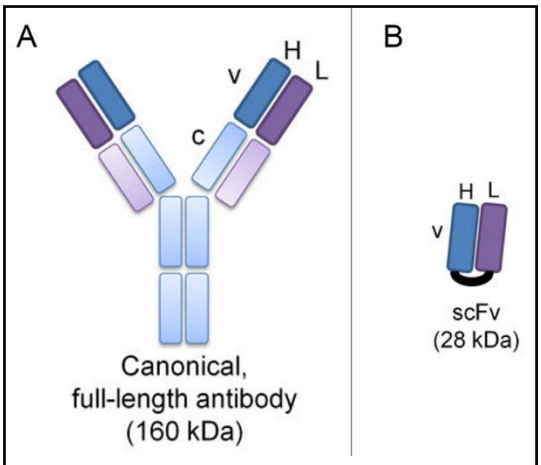

Figure 6. Structure of antibodies and scFvs. (A) Antibodies are heterotetramers composed of two identical heavy chains (blue) and two identical light chains (purple) joined by disulfide bonds. Each light chain contains a variable domain (dark purple) and one constant domain (light purple). The heavy chains also contain one variable (dark blue) and one constant (light blue) domain. (B) Single chain variable fragments are 28 kDa proteins composed of the variable domains of the light and heavy chains linked by a short peptide. Adapted from (87)

To target NCL, we have developed 4LB5, a fully human single-chain variable fragment (scFv) antibody. ScFvs are small molecules (26-28 kDa), derivative of an antibody (66). They are composed of the variable heavy (V_H) and variable light (V_L) domains of an antibody (the complete antigen binding site) connected via a short linker peptide (Fig. 6A-B) (67). These molecules bind to their target compounds with a similar affinity as the parent antibody (68).

Our compound, isolated by phage-display, binds with high affinity to the four RNA binding domains of NCL and disrupts the interaction between NCL and the miRNAs it regulates (48). In a previous study, 4LB5 was shown to bind to cell surface NCL on triple negative breast cancer cells and reduce cell survival and proliferation. This treatment resulted in decreased levels of miR-21, miR-221, and miR-222 (48).

Goals. This study aims to identify a regulatory pathway in skin cancer that explains, in part, NCL overexpression in skin cancers and validate 4LB5 as a compound for the treatment of these malignancies.

The specific aims of this study are to:

1. Investigate the post-transcriptional regulation of NCL
2. Evaluate 4LB5 as an antineoplastic tool for skin cancer treatment and its pathway of effect

Materials and Methods

Cell Cultures. SKMEL-147, human melanoma cells, and NL-145, murine melanoma cells, were provided by Dr. Christin Burd (OSU, Columbus, OH). SCC-13, human SCC cells, were provided by Dr. Amanda Toland (OSU, Columbus OH). All cell lines were cultured in DMEM with 10% (vol/vol) fetal bovine serum (FBS) and penicillin-streptomycin. Cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37℃.

Plasmids and Mutagenesis. The pcDNA3 flag p53 vector (FLAG-p53) was a gift from Thomas Roberts (Addgene plasmid # 10838) (69). The 3'-UTR of NCL mRNA (nucleotide 6 to nucleotide 419) containing the predicted miR-203 seed site, was cloned into the luciferase reporter gene plasmid psiCheck 2.0 (Promega): For, 5'-GGCCTCTAGAgtccctctgctttccctttt-3'; Rev, 5'-GGCCCTCAGAatttgtcctaaagccgctga-3'. The miR-203a-3p binding site (5'-ACATTTCA-3') was removed from the NCL 3'-UTR luciferase reporter plasmid through site directed mutagenesis (GENEART Site-Directed Mutagenesis System, Life-Technologies) according to the manufacturer's protocol.

Transfections, miRNAs, and siRNAs. Transfections were performed using Lipofectamine 2000 (Life Technologies) as described by the manufacturer. MiR-203a-3p miRNA (4464066), control miRNA (AM17110), TP53 siRNA (AM1331), NCL siRNA (4390824), and control siRNA (AM4611) were purchased from Life Technologies.

Luciferase Assay. In 12-well plates, SKMEL-147 cells were co-transfected with 20 ng of either the luciferase reporter plasmid containing the wild-type NCL 3'-UTR (psiCH-Ncl-WT) or the

mutated plasmid (psiCH-Ncl-MUT) and 100 nM of either control miRNA or miR-203a-3p. Luciferase and Renilla activity were assessed with the Dual-Light Luciferase system (Promega) according to the manufacturer's instructions at 48 h post-transfection. Luciferase activity was normalized to Renilla activity. All experiments were performed three times in triplicate, and the mean ± SD was reported.

Cell ELISA. 1.0 X 10^4 SKMEL-147 and NL-145 cells were plated in 96-well V-bottomed plates and incubated with increasing concentrations of 4LB5 for 2 h while shaking. Cells were washed with TPBS (0.1% Tween in PBS) and PBS before incubation with Penta-His HRP conjugate antibody (1000X, 3% BSA in PBS) for 1 h while shaking. Cells were again washed with TPBS and PBS. TMB was added to each well and the reaction was stopped with the addition of HCl in a 1:1 ratio. Absorbance at 450 nm was read. All experiments were performed in triplicate, and the mean ± SD was reported.

Protein Extraction, Western Blot, and Antibodies. Proteins were extracted using NIH lysis buffer with added phosphatase and protease inhibitors as previously described (48). Western blots were conducted according to standard procedures. Anti-NCL (D4C70), anti-pAKT (T308), anti-AKT (9272), and anti-GAPDH (14C10) were purchased from Cell Signaling. Other antibodies used included, anti-PTEN (138G6, Cell Signaling), anti-p53 (DO-1, Santa Cruz Biotechnology), anti-vinculin (4650, Cell Signaling), and anti-FLAG (F1806, Sigma).

RNA Extractions and Real-Time PCR. RNA was extracted using TRI Reagent (Sigma) according to the manufacturer's protocol. Real-Time PCR was performed as previously described

(48). Simultaneous quantification of RNU6 was used as a reference for miRNA quantification and GAPDH mRNA was used as a reference for mRNA quantification.

4LB5 Preparation and Treatments. 4LB5 is a recombinant protein produced from BL21DE3 E. Coli cells, as previously described (48). SKMEL-147 and NL-145 cells were control treated or treated with 25 nM-400 nM 4LB5 and harvested at different time points.

AlamarBlue and Colony Assay. AlamarBlue experiments were performed using the alamarBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's protocol on SKMEL-147 cells treated with 6.25-400 nM of 4LB5. For colony assay experiments, 200 SKMEL-147 or NL-145 cells were plated in 12-well plates with 10-100 nM of 4LB5 for 72 h. Cells were replenished with complete medium without 4LB5 and allowed to grow for 7 days. Then, cells were fixed with 1% glutaraldehyde in PBS and stained with Crystal violet.

UVR Assay. SKMEL-147 cells were exposed to 1 J/m^2 of UVB irradiation. At 6 h post-exposure, cells were collected. P53 and miR-203 expression were assessed via western blot and Real-Time PCR, respectively. Experiments were performed in duplicate and mean ± SD was reported.

Statistical Analysis. Student's t-test was used to determine significance (indicated as p-value). All error bars represent the SD of the mean. Data were considered statistically significant for $p < 0.05$.

Results

MicroRNA-203 downregulates NCL. Given the known role of miRNAs in tumorigenesis, we hypothesized that miRNAs may play a role in regulating NCL expression in skin cancers. Computational target predictions were used to identify miRNAs that could potentially target the 3'-UTR of NCL mRNA. TargetScan identified several miRNAs that could theoretically target NCL based on the presence of conserved sites in the 3'-UTR that matched the seed region of a miRNA (70). Of the predicted targets, we selected miR-203a-3p for further study because of its previously defined role in skin development and reported downregulation in skin cancers (57, 58, 59, 60).

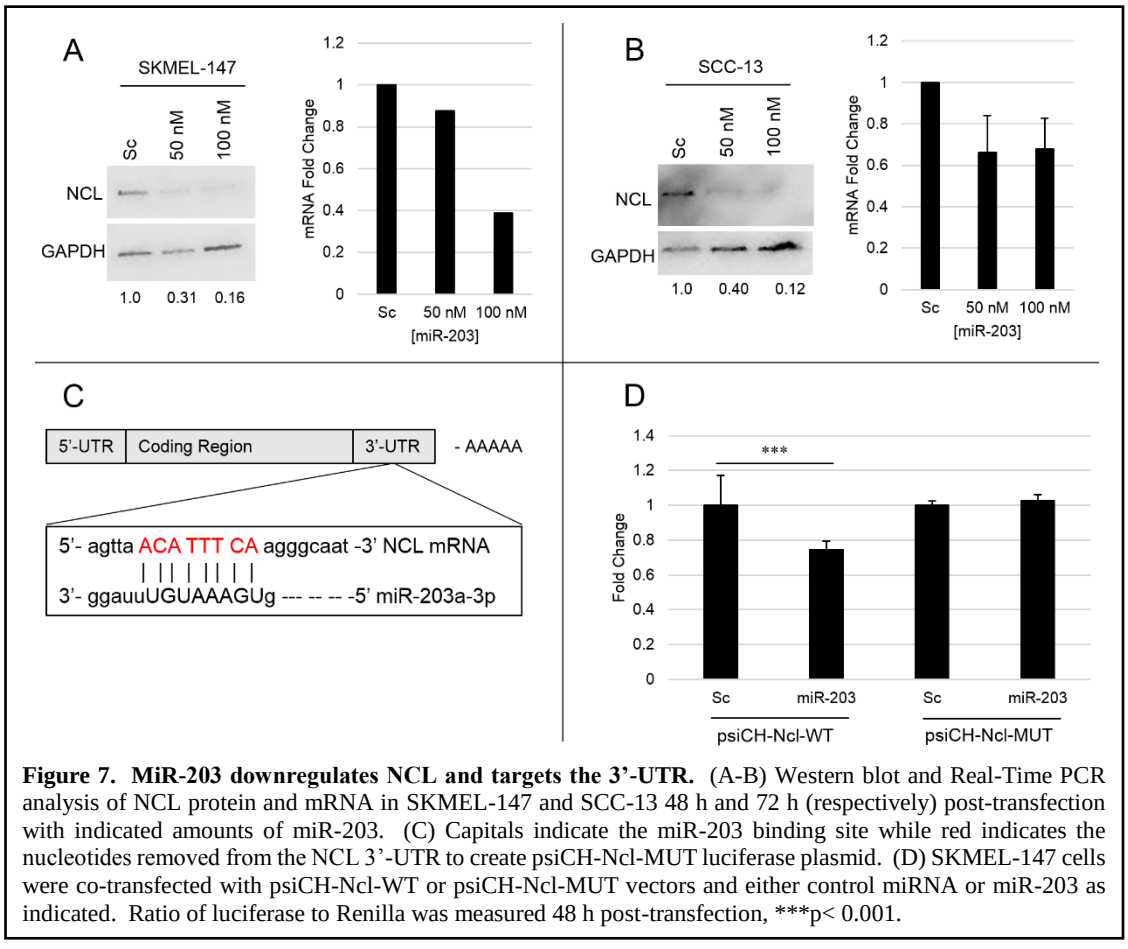

Figure 7. MiR-203 downregulates NCL and targets the 3'-UTR. (A-B) Western blot and Real-Time PCR analysis of NCL protein and mRNA in SKMEL-147 and SCC-13 48 h and 72 h (respectively) post-transfection with indicated amounts of miR-203. (C) Capitals indicate the miR-203 binding site while red indicates the nucleotides removed from the NCL 3'-UTR to create psiCH-Ncl-MUT luciferase plasmid. (D) SKMEL-147 cells were co-transfected with psiCH-Ncl-WT or psiCH-Ncl-MUT vectors and either control miRNA or miR-203 as indicated. Ratio of luciferase to Renilla was measured 48 h post-transfection, ***p< 0.001.

First, we investigated the effect of increasing miR-203 levels on NCL in human melanoma and SCC cell lines. SKMEL-147 and SCC-13 cells were transfected with 50 nM and 100 nM miR-203, and the levels of NCL protein and mRNA were assessed via Western blot and Real-Time PCR after 48 or 72 h. Fig. 7A-B shows reduced NCL protein and mRNA in miR-203 transfected cells in both cell lines. From this, we concluded that miR-203 had an inhibitory effect on NCL protein and mRNA levels.

Since the 3'-UTR of NCL mRNA contains a miR-203 binding site, we hypothesized that miR-203 was having its inhibitory effect by directly targeting NCL mRNA through an interaction with the 3'-UTR. To investigate this potential interaction, we utilized a luciferase reporter vector containing the 3'-UTR of NCL mRNA. The whole 3'-UTR was cloned into the dual-luciferase psiCheck 2.0 vector at the 3'-end of the luciferase coding region (psiCH-Ncl-WT). SKMEL-147 cells were then co-transfected with this vector and either scrambled control miRNA (Sc) or miR-203, and the ratio of firefly luciferase to Renilla was measured 48 h post-transfection. Additionally, to verify that the interaction of miR-203 and the NCL 3'-UTR was specific to the predicted miR-203 binding site, the binding site in the psiCH-Ncl-WT vector was removed through site directed mutagenesis to yield the psiCH-Ncl-MUT vector (Fig. 7C). As shown in Fig. 7D, in cells containing the wild type vector and miR-203, reduced luciferase activity was observed. Conversely, in cells containing the mutant vector, no difference between Sc and miR-203 transfected cells was seen. These data support the conclusion that miR-203 inhibits NCL activity through a site specific interaction with its 3'-UTR.

P53 regulates the expression of miR-203. Previous reports have identified p53 as a positive regulator of miR-203 expression (61). Based on these reports and the frequent loss or mutation of

p53 in SCC (17), we postulated that p53 regulates the expression of miR-203 in skin cancer. First, we investigated the effects of exogenous p53 expression on miR-203. To accomplish this, SKMEL-147 cells were transfected with a vector containing FLAG-tagged p53 (FLAG-p53). P53 protein levels were assessed by Western blot using an antibody against the FLAG tag while miR-203 levels were assessed by Real-Time PCR 48 h after transfection. As shown in Fig. 8A, miR-203 was expressed at higher levels in FLAG-p53 transfected cells compared to non-transfected controls.

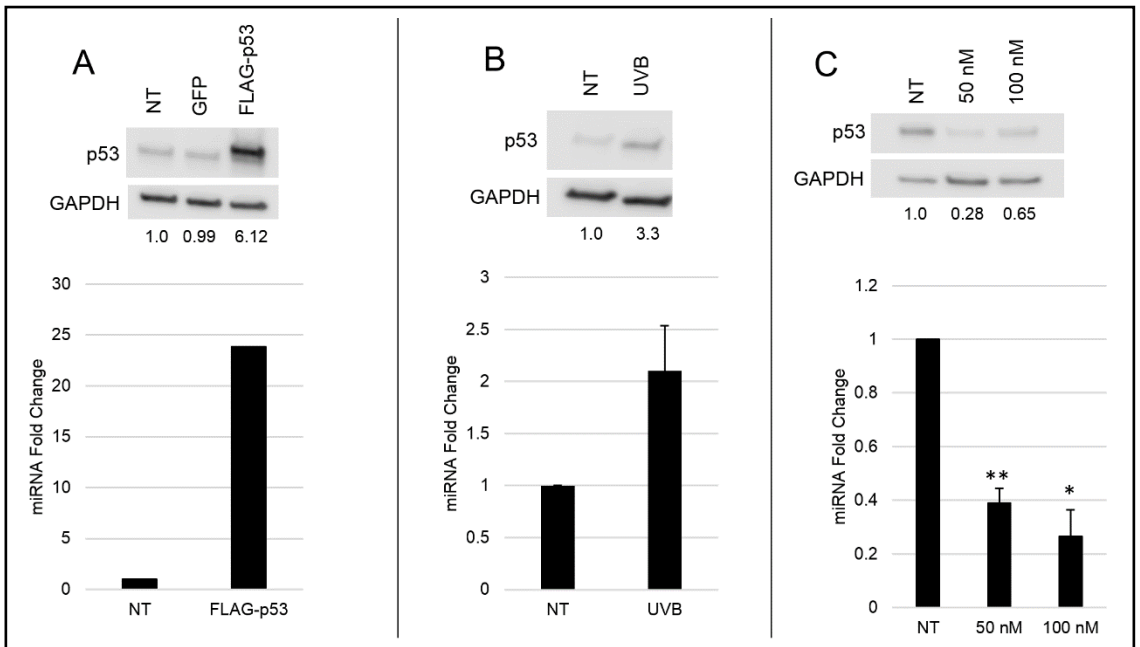

Figure 8. P53 regulates miR-203. (A) SKMEL-147 cells were transfected with FLAG-p53. P53 and miR-203 levels were assessed by western blot and Real-Time PCR, respectively after 24 h. (B) MiR-203 levels in response to p53 activation by UVR were observed 6 h after SKMEL-147 cells were exposed to 1 J/m² UVB. (C) SKMEL-147 cells were transfected with the indicated concentrations of si-p53. P53 downregulation and an increase in miR-203 expression were confirmed via western blot and Real-Time PCR, respectively, 72 h post-transfection, *p<0.05; **p<0.01.

We also explored the effects of p53 upregulation on miR-203 levels in response to UVB-induced DNA damage. SKMEL-147 cells were exposed to 1 J/m^2 of UVB radiation, and p53 upregulation at the protein level was assessed by Western blot, 6 h post-UV exposure. Effects on miR-203 levels were assessed using Real-Time PCR on total RNA extracts 6 h post-UV exposure.

Fig. 8B shows how in response to UVB exposure p53 protein levels increased in treated cells, as previously described (61). Notably, this upregulation was associated with an increase in miR-203 levels.

Additionally, we investigated the impact of p53 down-regulation on miR-203 expression. SKMEL-147 cells were transfected with 50 nM and 100 nM si-p53. After 72 h, p53 protein levels and miR-203 expression were assessed via western blot and Real-Time PCR, respectively. As shown in Fig. 8C, when p53 is decreased miR-203 expression is reduced.

Taken together, these data indicate that p53 positively regulates miR-203 in skin cancer cells.

4LB5 binds NCL on the surface of skin cancer cell lines.

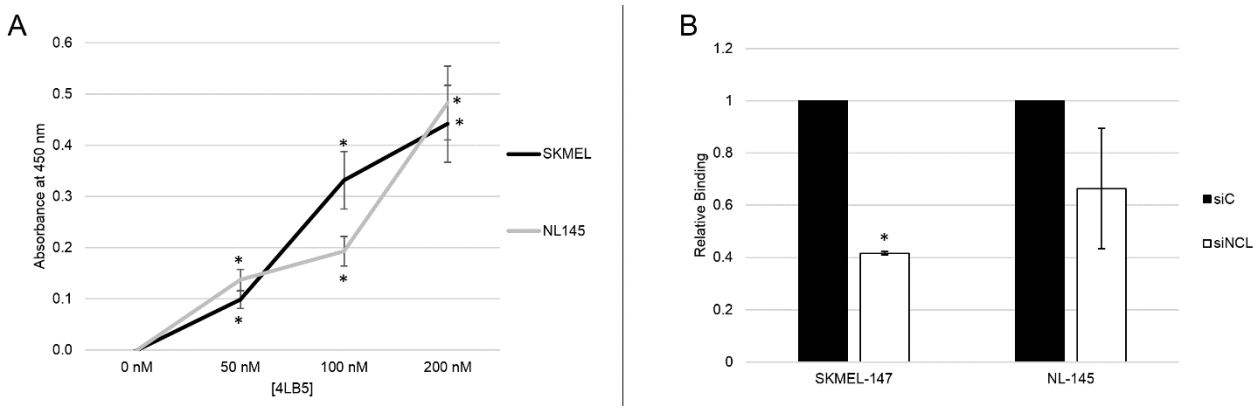

Figure 9. 4LB5 binds to the surface of melanoma cells. (A) Cell ELISA performed on SKMEL-147 and NL-145 melanoma cells using the increasing concentrations of 4LB5 indicated, *p<0.05. (B) SKMEL-147 cells were transfected with either siNCL or siControl (siC) RNA and ELISA was performed using 10nM of 4LB5, *p<0.05.

In cancer cells, NCL is frequently overexpressed resulting in oncogenic effects such as stabilization of BCL-2 and IL-2 mRNAs (71, 72). Additionally, NCL localization to the cell surface is found in cancer cells but not their normal counterparts (73, 74, 75), making it an attractive candidate for therapeutically targeting cancer cells. To this end, we have previously

isolated 4LB5, a single chain variable fragment with high affinity for the RNA binding domains of NCL that demonstrated antineoplastic activity in breast cancer models (48). To validate the use of 4LB5 as a tool for skin cancer therapy, we first confirmed that 4LB5 could bind NCL on the surface of melanoma cells. In order for 4LB5 to have a therapeutic effect on skin cancer cells, the scFv must bind to surface NCL. To assess binding, we performed a cell ELISA on SKMEL-147 and NL-145 cells incubated with 50-200 nM 4LB5. Fig. 9A shows that 4LB5 binding to both cell lines increased as the concentration of compound increased. To confirm that this binding was specific to cell surface NCL, we repeated this ELISA in cells transfected with either a control silencing RNA (siC) or a NCL specific siRNA (siNCL). Transfection with siNCL lowered total cellular NCL levels, consequently reducing surface NCL and resulting in reduced 4LB5 binding in these cells (Fig. 9B). These data indicate 4LB5 binds to the surface of human and mouse melanoma cells through a specific interaction with NCL.

4LB5 downregulates NCL-associated miRNAs in skin cancer. We have previously demonstrated that, once bound to surface NCL, 4LB5 can be internalized as NCL shuttles between

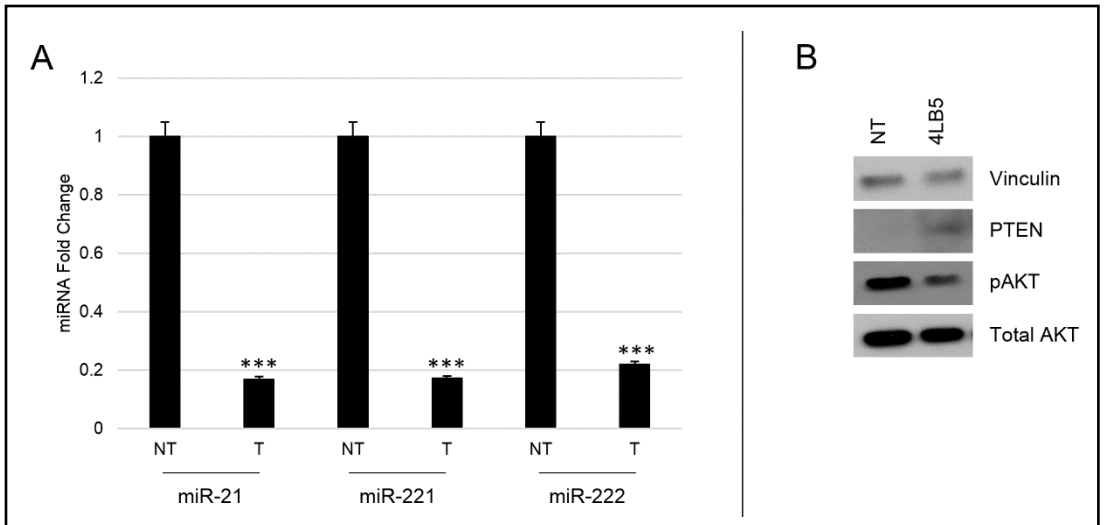

Figure 10. 4LB5 downregulates miRNAs and impacts PTEN and pAKT levels. (A) SKMEL-147 cells were treated with 100 nM of 4LB5 and RNA was extracted at 48 h. Levels of miR-21, -221, and -222 were assessed by Real-Time PCR, ***p<0.001. (B) SKMEL-147 cells were treated with 100 nM 4LB5. At 48 h, proteins were extracted and levels of PTEN and pAKT were assessed via Western blot.

cellular compartments (48). Internalized 4LB5 remains inside the cell where it continues to inhibit NCL (48). In previous breast cancer studies, 4LB5 treatment was shown to lower the levels of NCL-associated miRNAs by disrupting the protein's interaction with the miRNA (48), so we sought to investigate whether 4LB5 had similar effects in skin cancer. When SKMEL-147 cells were treated with 100 nM of 4LB5, the levels of miR-21, -221, and -222 were all significantly downregulated at 72 h post-treatment (Fig. 10A), indicating that, also in human melanoma cells, 4LB5 inhibits the NCL-dependent biogenesis of miR-21, -221, and -222.

Since NCL overexpression can lead to increased levels of miR-21, -221, and -222, we hypothesized that in cancer cells, this dysregulation leads to alterations in cellular pathways that allow cells to survive and proliferate. Previous reports have shown that miR-221/-222 are a negative regulator of PTEN (76). In normal cells, PTEN negatively regulates the activation of the AKT pathway, preventing excessive cellular proliferation (77, 78, 79), but in skin cancer, PTEN is frequently lost (80). We sought to determine if treatment with 4LB5 could reverse the loss of PTEN by lowering the levels of miR-221/-222. In SKMEL-147 cells treated with 100 nM of 4LB5 for 48 h, PTEN protein was increased while AKT activation as measured by pAKT levels, was decreased (Fig. 10B) compared to non-treated cells. In summary, these results indicate that AKT activation can be, at least in part, reduced through upregulation of PTEN, by downregulating NCL-dependent microRNAs via treatment with 4LB5.

4LB5 inhibits cellular proliferation. To determine whether the downregulation of NCL-associated miRNAs and subsequent upregulation of PTEN was leading to reductions in proliferation, we assessed the effect of 4LB5 treatment on the proliferation of skin cancer cells. First, we performed an alamarBlue assay on SKMEL-147 cells treated with increasing

concentrations of 4LB5 (0-400 nM). As shown in Fig. 11A, cellular proliferation decreased with higher treatment concentrations.

Effects on proliferation were also assessed through colony assay. SKMEL-147 and NL-145 melanoma cells were plated at sub-confluent levels and treated with 10-100 nM 4LB5. After 72 h of treatment, media was changed and cells were allowed to grow for 7 days, at which point colony number was counted after crystal violet staining. In both SKMEL-147 and NL-145 cells, proliferation compared to non-treated controls decreased significantly at the higher treatment concentrations (Fig. 11B).

These observations indicate that 4LB5 inhibits cellular proliferation in both human and mouse melanoma cells.

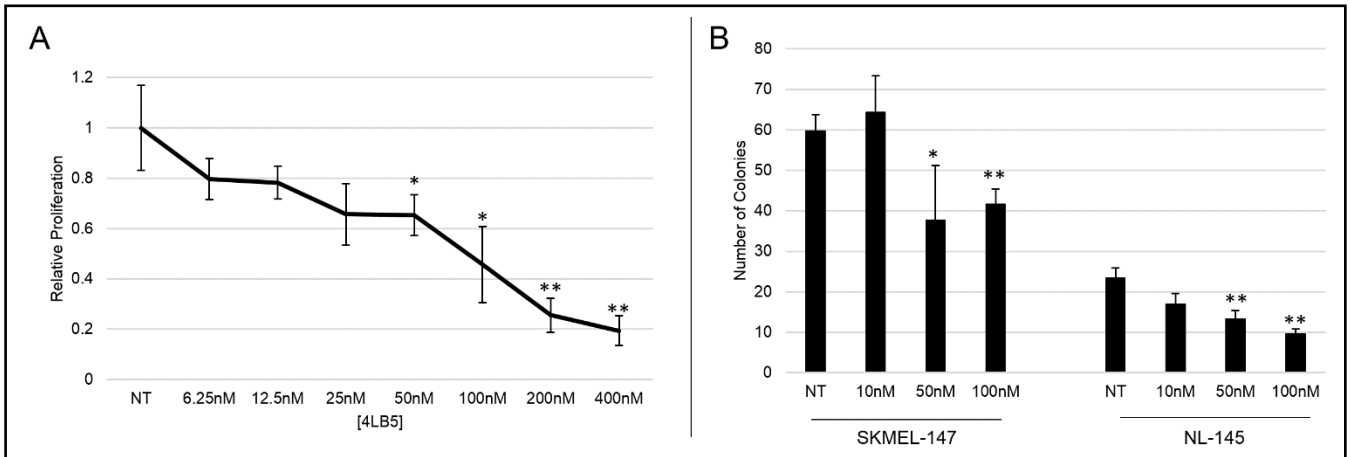

Figure 11. 4LB5 inhibits proliferation. (A) SKMEL-147 cells were plated in 96-well plates and treated with the indicated increasing concentrations of 4LB5. Proliferation was assessed by alamarBlue assay 48 h after treatment, *p<0.05; **p<0.01 compared to non-treated controls. (B) Cells were plated in 6-well plates and treated with the indicated concentrations of 4LB5. Colonies were stained with crystal violet and counted 7 days after treatment, *p<0.05; **p<0.01 compared to non-treated controls.

Discussion

Skin cancer represents a significant and growing burden on the health care system (1). It is the most commonly diagnosed malignancy in the United States, and its incidence is expected to increase in the future due to rising exposure to UV radiation via sunlight (4). Current treatment options for the most aggressive form, malignant melanoma, are limited and often ineffective in the later stages of the disease (6). Thus, there is a need to design and develop new therapeutics. NCL represents an attractive therapeutic target because of its cancer-cell specific localization to the plasma membrane and involvement in the biogenesis of oncogenic microRNAs (27, 34, 48).

The mechanism of NCL upregulation in cancer cells is not well defined, but recent reports have provided evidence of regulation by miR-206, -194, and -494 in breast cancer (81, 82). Through computational target predictions, we identified that the NCL 3'-UTR also contained target sequences for miR-203 (70). In SKMEL-147 and SCC-13 cells, exogenous expression of miR-203 led to significant decreases in NCL expression. Since in transfected cells, decreases in NCL mRNA were observed, it is likely that this interaction causes reductions in NCL expression by destabilizing the mRNA rather than by affecting translation efficiency. Luciferase reporter assays confirmed that miR-203 directly targets the 3'-UTR.

MiR-203 expression is frequently lost in skin cancers, and its loss is associated with increased tumor aggressiveness and stage (57, 58, 59, 60). Previous reports have linked miR-203 expression with p53 expression (61), and we sought to verify this relationship in skin cancer cell lines. In line with previous reports, exogenous expression of p53 in SKMEL-147 cells resulted in elevated miR-203 expression. Additionally, activation of p53 in SKMEL-147 cells by exposure to UVB radiation led to increased miR-203 levels. In contrast, transfection of these cells with a p53 silencing RNA resulted in miR-203 reduction. The specific mechanism of interaction between

p53 and miR-203 is still unknown, and previous studies have failed to identify an interaction between the miR-203 promotor and p53 (83), implying that p53 may influence miR-203 expression through an indirect pathway.

Taken together, our results support a pathway from DNA damage to increased NCL expression via p53 and miR-203. As depicted in Fig. 12A, in response to DNA damage, p53 expression and activation increases resulting in higher levels of miR-203 through a yet to be identified mechanism. Increased expression of miR-203 destabilizes NCL mRNA and prevents overexpression of the protein, thus preventing the overexpression of oncogenic miRNAs. However, in cells with mutated or abrogated p53, miR-203 upregulation is not accomplished and NCL expression increases unchecked.

Since NCL is overexpressed and present on the cell surface of skin cancer cells, we sought to determine whether NCL targeting was a viable strategy for skin cancer treatment. Previously, we developed 4LB5, a scFv targeted to the 4 RNA binding domains of NCL, to target cell surface NCL (48). In breast cancer, we demonstrated that the antineoplastic effects of 4LB5 were achieved, at least in part, through the downregulation of three oncogenic miRNAs, miR-21, -221, and -222 (48). In melanoma cell lines, 4LB5 bound specifically to cell surface NCL and this binding led to significant reductions in miR-21, -221, and -222. Since these miRNAs are negative regulators of PTEN, which is commonly lost in skin cancers, we evaluated the effects of 4LB5 treatment on PTEN and its downstream target, AKT. In response to treatment, PTEN levels in SKMEL-147 cells were increased while the activation of AKT, as measured by pAKT levels, were decreased. Additionally, as shown by colony and alamarBlue assays, the proliferation of treated SKMEL-147 and NL-145 cells was significantly reduced. These data support 4LB5 as a promising

tool for skin cancer treatment and NCL targeting as a valid approach in the development of cancer therapeutics.

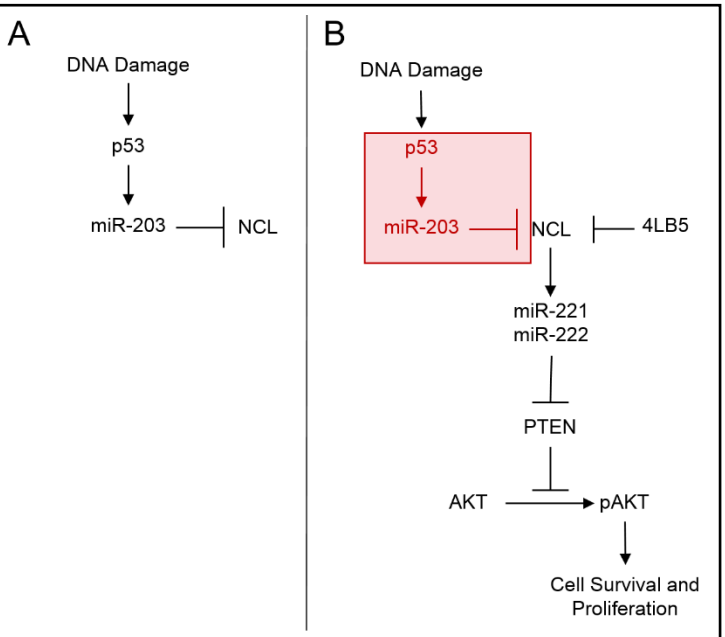

Figure 12. Regulation of NCL in response to DNA damage. (A) In normal cells, DNA damage due to UVB results in the upregulation of p53 which increases the expression miR-203. This miRNA inhibits NCL and prevents its overexpression. (B) In cells with lost or mutated p53, miR-203 is not upregulated in response to DNA damage and NCL is over expressed leading to an increase in miR-221/-222 which allows the cell to survive and proliferate by inhibiting PTEN and hyper-activating the AKT pathway. Treatment with 4LB5 combats NCL over-expression by preventing the biogenesis of miR-221/-222 allowing increased PTEN expression and lower proliferation.

Overall, we propose that when p53 is lost or mutated, cells lose one of the mechanisms responsible for NCL regulation, resulting in an overexpression of this protein (Fig. 12B). Overexpression of NCL leads to higher levels of miR-21, -221, and -222 which alter the functions of numerous pathways. In one of these pathways, PTEN expression is suppressed allowing for the hyper-activation of AKT, leading to increased cellular proliferation and contributing to the development of skin cancers. However, with 4LB5, we can target this pathway downstream of the initial mutation to restore PTEN expression and inhibit proliferation.

Future investigation of the effects of miR-203 expression on the cellular localization of NCL is required to determine if miR-203 could be a marker for 4LB5 sensitivity in skin cancers. Since the mechanism of miR-203 regulation by p53 is still unknown, studies should also focus on this area. Association studies in melanoma patients between p53 mutations and NCL overexpression, as well as expression levels of miR-21, -221, -222, and -203 could offer additional insights into the mechanism of NCL regulation in skin cancer. In summary, we have identified

and described a potential pathway of NCL overexpression in skin cancer cells and demonstrated that the effects of this pathway can be abrogated by targeting NCL with 4LB5.

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